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Diversification of shrub frogs (Rhacophoridae, *Pseudophilautus*) in Sri Lanka – Timing and geographic context

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ABSTRACT

Pseudophilautus comprises an endemic diversification predominantly associated with the wet tropical regions of Sri Lanka that provides an opportunity to examine the effects of geography and historical climate change on diversification. Using a time-calibrated multi-gene phylogeny, we analyze the tempo of diversification in the context of past climate and geography to identify historical drivers of current patterns of diversity and distribution. Molecular dating suggests that the diversification was seeded by migration across a land-bridge connection from India during a period of climatic cooling and drying, the Oi-1 glacial maximum around the Eocene-Oligocene boundary. Lineage-through-time plots suggest a gradual and constant rate of diversification, beginning in the Oligocene and extending through the late Miocene and early Pliocene with a slight burst in the Pleistocene. There is no indication of an early-burst phase of diversification characteristic of many adaptive radiations, nor were there bursts of diversification associated with favorable climate shifts such as the intensification of monsoons. However, a late Miocene (8.8 MYA) back-migration to India occurred following the establishment of the monsoon. The back migration did not trigger a diversification in India similar to that manifest in Sri Lanka, likely due to occupation of available habitat, and consequent lack of ecological opportunity, by the earlier radiation of a sister lineage of frogs (*Raorchestes*) with similar ecology. Phylogenetic area reconstructions show a pattern of sister species distributed across adjacent mountain ranges or from different parts of large montane regions, highlighting the importance of isolation and allopatric speciation. Hence, local species communities are composed of species from disparate clades that, in most cases, have been assembled through migration rather than *in situ* speciation. Lowland lineages are derived from montane lineages. Thus, the hills of Sri Lanka acted as species pumps as well as refuges throughout the 31 million years of evolution, highlighting the importance of tropical montane regions for both the generation and maintenance of biodiversity.

1. Introduction

Mountainous islands in the tropics are often hotspots of endemism and diversity but the processes underlying this pattern are poorly known. The isolation of islands contributes to their high endemism, but the processes of diversification within islands are complex. Interaction between broad-scale climate fluctuations and the topographic complexity of mountainous tropical regions may promote the accumulation and persistence of species richness in a variety of ways (Fjeldsa et al., 2012; Graham et al., 2004; Smith et al., 2014). Habitat variation from low to high altitudes provides the opportunity for speciation due to divergent selection across ecological gradients (Cadena et al., 2011;

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Graham et al., 2004; Ogden and Thorpe, 2002; Smith et al., 2001). The high relief of montane regions, with cool, wet upland zones often separated by warmer and drier valleys or lowlands, provides the opportunity for allopatric divergence of cool-wet-adapted species on isolated mountain ranges (Bossuyt et al., 2004; Cadena et al., 2011; Kozak and Wiens, 2006; Meegaskumbura and Manamendra-Arachchi, 2005; Moreau, 1966; Schneider et al., 1998; Schneider and Moritz, 1999; Vijayakumar et al., 2016). In addition, montane regions can provide climatically stable areas where suitable habitats persist through global and regional climate fluctuations (Carnaval et al., 2009, 2014; Fjeldsa et al., 2012; Schneider and Williams, 2005), thus contributing to the buildup of species richness. Teasing apart the relative contributions of climate, geography and different evolutionary processes on diversification is a longstanding challenge. Recent advances in phylogenetic and historical biogeographic analyses, however, provide the opportunity to gain insight into the pattern and process of diversification that allow assessment of the relative effects of geology, geography, dispersal and climate on diversification, persistence and species richness.

Rhacophorid frogs of the genus *Pseudophilautus* in Sri Lanka represent a spectacular, endemic diversification of anurans on a mountainous continental island (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura et al., 2002; Meegaskumbura and Manamendra-Arachchi, 2005; Meegaskumbura, 2007). Seventy-six extant species are recognized at present, based on both morphological and molecular data (AmphibiaWeb, 2018), representing a five-fold increase from the approximately 15 species recognized less than 20 years ago. The remarkable diversification of these frogs appears to have been aided by their direct-developing reproductive mode, which allows them to breed in moist habitats away from bodies of standing or running water, even in relatively dry areas (Bahir et al., 2005). Similar diversifications have occurred in other direct-developing anuran genera such as the Terrarana clade of the New World (Hedges et al., 2008) and the Indian bush frogs, *Roarchestes* (Vijayakumar et al., 2016), and direct development appears to be a key innovation that facilitates diversification (Blackburn et al., 2013; Bossuyt and Milinkovitch, 2001; Setiadi et al., 2011). In Sri Lanka, *Pseudophilautus* species occupy several habitat types ranging from open grasslands, rock substrates along streams, rainforest canopy, shrubs, leaf litter and anthropogenic habitats, and most are confined to higher elevations of the wet, montane region of the island's southwestern quarter (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura et al., 2007, 2009, 2012; Meegaskumbura and Manamendra-Arachchi, 2005, 2011). The three main mountain ranges of this region constitute ecological islands of cool, wet habitat separated by low, warm and often drier valleys.

Key questions regarding diversification of *Pseudophilautus* in Sri Lanka following the initial colonization concern the geographic context of speciation and the impact of climate change over time. Are species assemblages in each mountain range monophyletic or is there evidence for dispersal between mountain ranges? Did diversification occur as an early burst or as a succession of events of geographic range expansion and subsequent fragmentation associated with climatic cycles? Are there geologic and/or climatic events that had overarching impacts and shaped the radiation over time?

Here we use a multi-gene, time-calibrated phylogeny of nearly all extant species of Sri Lankan and Indian *Pseudophilautus* to estimate rates of diversification over time in relation to key geologic and geographic events and to evaluate hypotheses regarding the drivers of patterns of diversity.

2. Materials and methods

2.1. Phylogeny

2.1.1. Tissue collection, extraction, PCR amplification and sequencing

This analysis included 67 putative species of *Pseudophilautus* in Sri Lanka; 2 species of *Raorchestes*, the sister group of *Pseudophilautus* (Abraham et al., 2013)—*R. charius* and *R. signatus*; the Indian ingroup species *Pseudophilautus wynaadensis*, *P. amboli* and *P. kani*; and 1 or 2 representatives from each of the other rhacophorid genera (S1 Table). The trees were rooted using 4 species of Mantellidae: *Mantella aurantiaca*, *M. madagascariensis*, *Mantella* sp. and *Mantidactylus grandidieri*. Previous studies suggest that this family is the closest sister-group of Rhacophoridae (Li et al., 2013; Meegaskumbura et al., 2015). The 67 putative species of *Pseudophilautus* mentioned above were included to derive as complete a phylogeny as possible for the Sri Lankan diversification. Of these species, 48 have been formally described and named, whereas 19 await formal description. Tissue samples were collected in the field, along with locality data. (See S1 Table for taxon sampling, gene sampling and distributional data, as well as reference numbers.)

For molecular systematic analysis, DNA was extracted from ethanol-preserved tissues using Qiagen tissue extraction kits following manufacturer's protocols. A total of 2907 base pairs (bp) were sequenced, which included two mitochondrial ribosomal RNA gene fragments (*12S rRNA* and *16S rRNA*; ca. 1504 bp) and one large fragment of the nuclear gene *Rag-1* (ca. 1403 bp). Portions of the *12S rRNA, 16S rRNA* and *Rag-1* genes were amplified by PCR and sequenced directly using dye-termination cycle sequencing. Primer sets and protocols for both PCR and sequencing are described in detail in the Supplementary Material (S1 Text).

2.1.2. Sequence alignment

Chromatograms were edited visually using 4peaks (v. 1.7.1). Mitochondrial *16S rRNA* and *12S rRNA* gene fragments were initially aligned using ClustalW as implemented by MEGA v.6.0 (Tamura et al., 2013); regions for which we had low confidence in positional homology were removed from the analysis. Nuclear *Rag-1* gene sequences were also aligned using MEGA v.6.0 with translated amino-acid sequences. The complete concatenated dataset included 94 taxa (duplicated taxa were pruned from the dataset) with a total of 2177 bp (*16S rRNA*, 464 bp; *12S rRNA*, 310 bp; *Rag-1*, 1403 bp).

2.1.3. Phylogenetic analyses

Tree topology was inferred using maximum likelihood (ML) analyses that were performed for both partitioned and unpartitioned datasets and also for each gene individually. The dataset was partitioned into specific gene regions by specifying character sets (charset 16S rRNA = 1–464; charset 12S rRNA = 465–775; charset Rag-1 = 776–2178). The partitioned dataset was used for the phylogenetic analyses discussed in detail. The best-fitting nucleotide substitution model for each dataset was chosen using jModelTest v.2.1.4 (Darriba et al., 2012; Guindon and Gascuel, 2003). We used the Cipres Science Gateway Server (Miller et al., 2010) to run the ML analysis using RAxML 7.2.8 (Stamatakis, 2006)) on the partitioned dataset. The model of evolution, as selected from the jModelTest (GTR + I + G), was given with all parameters estimated along with 1000 bootstrap pseudoreplicates (model parameters for each dataset are given under Supplementary Information). Subsequently, the unpartitioned dataset and the three genes individually were also analyzed using RAxML.

Clade support was assessed using ML bootstrapping and Bayesian posterior probabilities (PP). Bootstrapping was done in an ML framework using RAxML 7.2.8 (Stamatakis, 2006), with 1000 RAxML searches and 1000 iterations. BEAST v.1.8 (Drummond and Rambaut, 2007) was used to infer trees with a Bayesian criterion and to assess posterior probability at each node. The Yule model was given as the tree prior; the model of sequence evolution was the same as that used in the ML analysis. Four Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were run for 10 million generations and for two consecutive runs. Burnin was defined by observing the log-output file in Tracer v.1.6 (Drummond et al., 2012); 90% of the post-burnin trees were analyzed using TreeAnnotator. Consensus trees using unpartitioned datasets for ML and Bayesian analyses are given as Supplementary Information, as are log-output files.

Analyses of partitioned and unpartitioned datasets yielded similar topologies but slightly different branch lengths. However, since the partitioned data set provides greater node support, we use this dataset and the corresponding tree for timing-related analyses and geographic reconstructions.

2.2. Divergence time

To examine the temporal context of divergence and to correlate it with geological and climatic events, we estimated divergence time among lineages on the partitioned dataset by using BEAST v.1.8 (Drummond and Rambaut, 2007). The Yule model was given as the tree prior, the GTR + I + G model of evolution specified, and other parameters estimated for all gene partitions. Over 10 million generations of MCMC simulations were run, with one tree saved per 1000 generations. The analysis was repeated twice: once using the lognormal relaxed clock (uncorrelated) and a second time using the default strict clock (with random starting trees). The lognormal relaxed clock model showed the greatest fit and was used in all subsequent analyses. We calibrated the tree using the split between Mantellidae and Rhacophoridae, a reference point used in previous studies (Li et al., 2013; Roelants et al., 2004), using the fossil calibration date (73.1 ± 19 MYA) designated by Bossuyt and Milinkovitch (2001).

2.3. Lineage-through-time (LTT) analysis

We analyzed the accumulation of lineages through time to discern the tempo of diversification in the radiation (Harmon et al., 2007) and to assess its possible relation to climatic and geographic events. Lineage-through-time plots were constructed by plotting the log number of lineages against the log divergence time. The plots were generated in R v.1.0 using the packages *ape*, *laser* and *geiger* (Paradis, 2006; Rabosky, 2006). A null model was also developed wherein the tree was simulated with a total of 94 taxa and assuming a constant rate of speciation across the lineages (under a pure birth model) with no extinctions. A Monte Carlo Constant Rate test (MCCR; Pybus and Harvey, 2000) was used to calculate the gamma (γ) statistic (using the *laser* package; Rabosky, 2006), which compares the distances between the tree-root and the midpoint to the distances between the midpoint and tips. If $\gamma < 0$, the radiation shows an early, rapid speciation event; if $\gamma = 0$, the radiation undergoes a constant diversification rate; and if $\gamma > 0$, the diversification rate increases towards the present (Pybus and Harvey, 2000).

2.4. Geographic reconstruction

To evaluate the geographic origins and movements of lineages over time, we estimated the ancestral geographic state at nodes in the phylogeny using both ML (Lagrange v. 1.5; Ree and Smith, 2008) and Bayesian frameworks (BEAST v.2.0; Bouckaert et al., 2014).

The Lagrange analysis included the following steps: the partitioned dataset and the chronogram resulting from the BEAST dating analysis were uploaded to the Lagrange Configurator. Sri Lankan species inhabiting elevations 700 m and above were segregated by the three main mountain ranges: Central Hills (C), Knuckles Hills (K) and Rakwana Hills (R). Species found below 700 m were placed in a single category: Lowlands wet (LL). Indian species were coded as (IND) and the remaining rhacophorids + outgroup were coded as undesignated (U), as their distributional data were not included in the analysis. Based on available data on the distribution of Sri Lankan species (AmphibiaWeb 2018; Bahir et al., 2005; Meegaskumbura and Manamendra-Arachchi, 2005, 2011; Manamendra-Arachchi and Pethiyagoda, 2006; Meegaskumbura et al., 2007, 2009, 2012; Wickramasinghe et al., 2013), the following dispersal routes were evaluated: Central-Rakwana, Central-Knuckles, Central-Lowlands wet, Central-India, Knuckles-Rakwana, Knuckles-Lowlands wet, Knuckles-India, Rakwana-Lowlands wet, Rakwana-India and India-Lowlands wet. Assuming that *Pseudophilautus* have poor dispersal abilities over non-adjacent areas, low probability values (0.1) were assigned to four routes: Central-India, Rakwana-India, Knuckles-India and Knuckles-Rakwana. The maximum number of areas reconstructed at a node was fixed as two.

Reconstructions using the Bayesian framework were done using the Standard-BEAST method as implemented in BEAST v. 2.2.0 (Bouckaert et al., 2014). Codings for the different regions (C, R, K and U) were defined in the same way as in the Lagrange analysis. The input XML file was created using BEAUti v. 2.0 using the partitioned dataset. The lognormal-relaxed (uncorrelated) clock was adopted, a Yule speciation model was given as the tree prior, and a gamma prior distribution was used for the discrete trait. The run time was ten million generations and the MCMCMC analysis was performed on four chains. The burnin point was determined by observing the log file on Tracer v1.6 (Rambaut et al., 2014) and 90% of post-burnin trees were summarized in TreeAnnotator.

3. Results

3.1. Phylogeny

Phylogenetic analysis strongly supports the monophyly of *Pseudophilautus,* with the clade of *Raorchestes* + *Mercurana* as its sister group. Internal nodes of the *Pseudophilautus* clade are for the most part well supported. Building on these relationships, we identify six component clades (Fig. 1, clades A–F). Clade A mostly comprises species that inhabit higher elevations of the three major mountain ranges. Clade B has a broad representation of species from low to high elevations. Clade C is mostly an assemblage of species from low to high elevations and includes some of the largest species of *Pseudophilautus*. Clade D harbors frogs from low to high elevations. Clade E is represented mostly by low-to-mid-elevation species, but it also includes a clade harboring three Indian species. Clade F comprises some of the smallest frogs, which inhabit the higher elevations of the three major mountain ranges. The genera *Buergeria*, *Liuixalus*, (*Theloderma* + *Nyctixalus*), ((*Rhacophorus* + *Ghatixalus* + *Polypedates* + *Taruga*) + (*Feihyla* + "*Feihyla*" + *Chiromantis*)), (*Gracixalus*), (*Philautus*), (*Kurixalus*) and (*Beddomixalus*), (*Mercurana* + *Roarchestes*), and (*Pseudophilautus*) formed sequential sister clades (Fig. 1). *Mercurana* or *Raorchestes* is the sister group of *Pseudophilautus*; the node uniting (*Raorchestes* + *Mercurana*) is poorly supported by ML bootstrap (64) but highly supported by Bayesian posterior probability (> 95). Topologies of the consensus trees of Bayesian and ML analyses are similar in their assembly of major clades (Figs. 1, 2).

3.2. Divergence-time and Lineage-through-time analyses

Divergence-time analysis, calibrated using the rhacophorid-mantellid split (dated at 67.8 MYA; Li et al., 2013), suggests that the split between *Pseudophilautus* and *Raorchestes* occurred ca. 31 MYA and that the most recent common ancestor (MRCA) of extant *Pseudophilautus* dates to ca. 25 MYA (95% PI = 21.93–45.14; Fig. 2; Table S4). Major clade diversification seems to have occurred during the early-to-mid Miocene, from 21.3 to 13.4 MYA (Fig. 2A). However, most extant *Pseudophilautus* species evolved during the late Miocene and Pliocene, and several currently recognized species evolved during the early Pleistocene (ca. 1 MYA). The back-migration of *Pseudophilautus* to India occurred during the late Miocene (Fig. 2C).

Lineage-through-time plots suggest that diversification within *Pseudophilautus* occurred gradually throughout the last 31 million years (Fig. 2B); there is no indication of an early burst of speciation during the initial colonization of the island. The log-null model (a pure birth model with constant diversification rates across the lineages) is close to the actual cumulative lineage curve (Fig. 3). The γ statistic value of

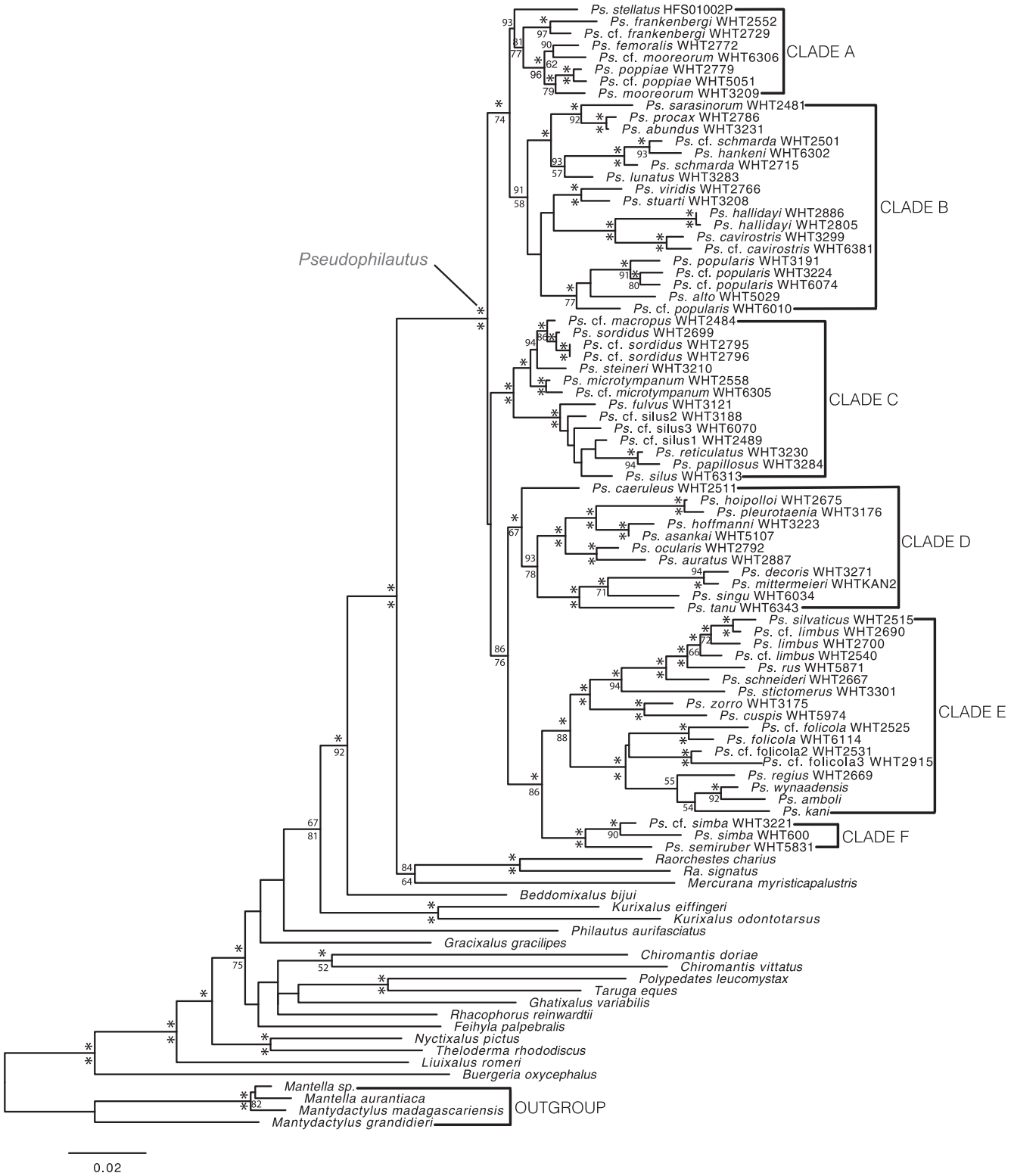

Fig. 1. Multi-gene (*12S rRNA*, *16S rRNA* and *Rag-1*) partitioned ML phylogram of rhacophorid genera with an emphasis on Sri Lankan-Indian species of *Pseudophilautus*. The tree includes a total of 94 taxa and is rooted with four mantellid species. The monophyletic radiation of *Pseudophilautus* comprises six clades, A–F. Its sister group is a clade containing the Indian genera *Mercurana* and *Raorchestes*. This topology forms the basis of all subsequent analyses. Asterisks (*) above and below nodes indicate > 95% Bayesian posterior probabilities and ML bootstrap values, respectively.

1000 MCCR simulations, 2.91, suggests there was a low magnitude of diversification after the midpoint of the tree. Based on the slight deviation from the null model (Fig. 3), the slightly positive value can be attributed to the lineage's late diversification (late Pliocene and Pleistocene).

3.3. Geographic area reconstruction

Ancestral-area reconstructions using Lagrange (ML approach) and BEAST (Bayesian approach) yield similar patterns. For brevity, we depict the Lagrange analysis (Fig. 2); the BEAST reconstruction is

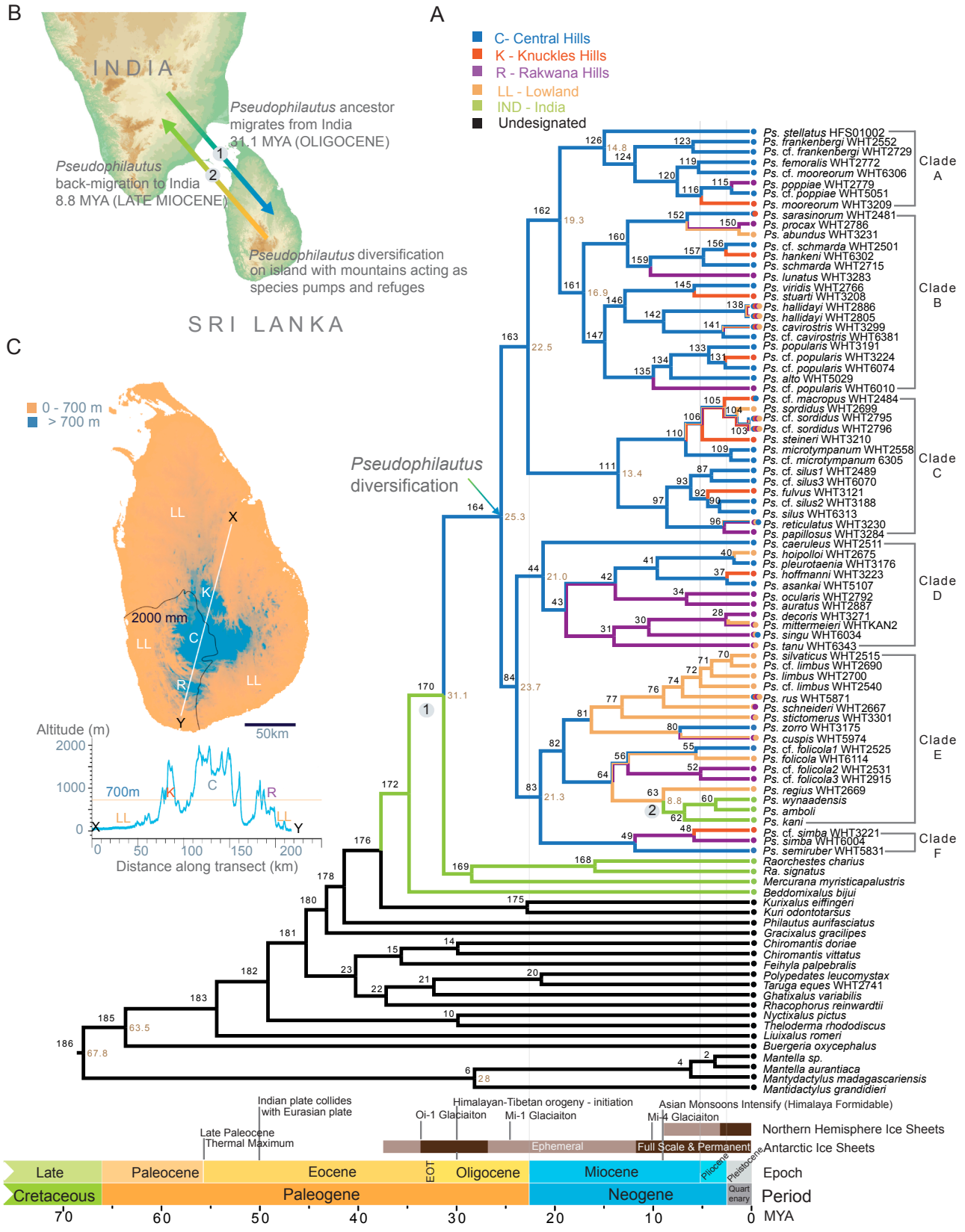

Fig. 2. Timing and context of *Pseudophilautus* diversification. (A) Time-calibrated tree of the dating analysis using BEAST and output from the Lagrange analysis for area reconstruction. Node numbers from the Lagrange analysis are set in black font (see also S4 Table). Numbers set in brown font at several major nodes are dating estimates in MYA (error intervals in Table S3). Key events include the common ancestor of *Pseudophilautus* migrating to Sri Lanka over exposed land bridges during the late Oligocene (perhaps at the Oi-1 glacial maxima); a late-Oligocene-to-Pleistocene diversification on the island, which may have been facilitated by the Himalayan-Tibetan orogeny and correlated intensification of the monsoons; and a late Miocene back-migration of *Pseudophilautus* to the mainland, likely across land bridges during a glacial period (perhaps at the Mi-4 glacial maxima). (B) Depiction of *Pseudophilautus* movements between India and Sri Lanka. (C) A map of Sri Lanka depicting the three main mountain ranges (C, Central Hills; K, Knuckles Hills; R, Rakwana Hills), Lowlands (LL) and India (IND). The 2000 mm rainfall contour demarcates the wet zone in Sri Lanka. X–Y line depicts the altitudinal transect across the three main mountains.

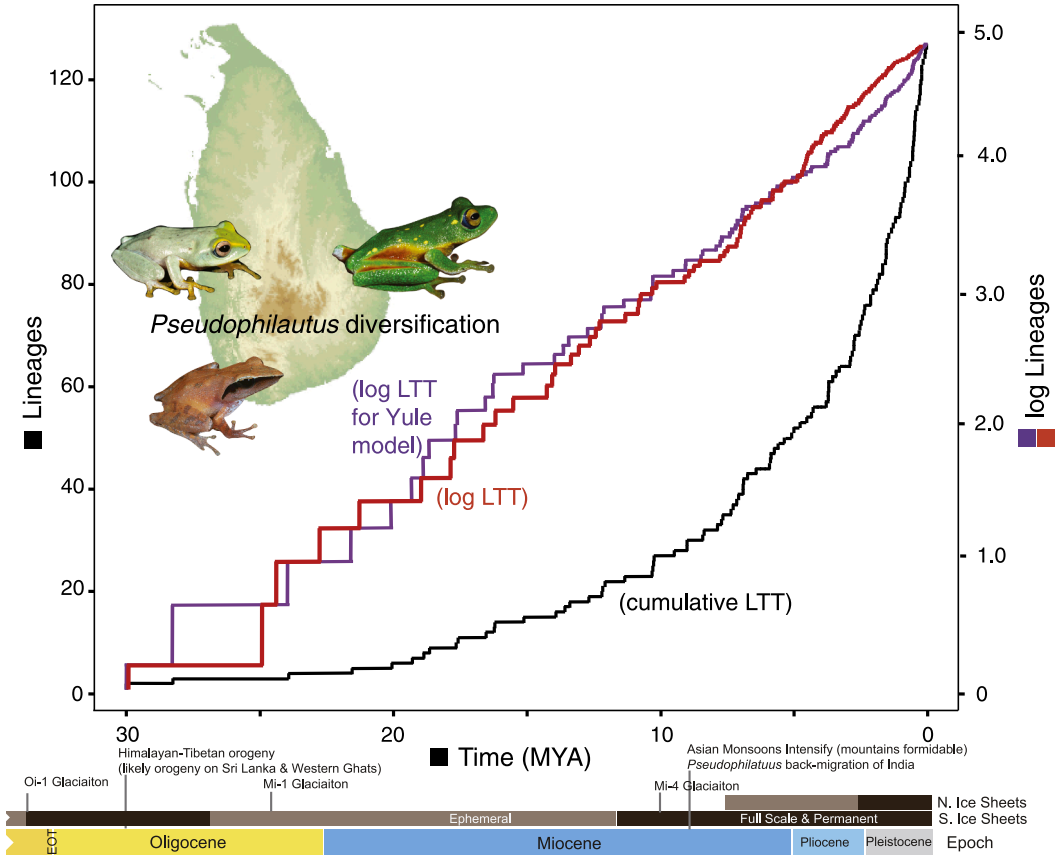

Fig. 3. Rates of diversification of *Pseudophilautus*. A cumulative lineage-through-time plot indicates a slow diversification rate through time without an early burst of lineages. However, a slight late burst is suggested with a gamma (γ) value of 2.91, which might be attributable to Pleistocene and Pliocene diversification. The null (Yule) model is indicated by the purple line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presented in Supplementary Information (S1 Fig.). Reconstruction of ancestral geographic area places the MRCA of the *Pseudophilautus* radiation in the mountainous Central Hills (Fig. 2A; node 164). Subsequent colonization of areas outside the Central Hills has occurred repeatedly in each major clade. Ancestral-area reconstruction also shows a frequent pattern of sister species distributed across the three mountain regions, as well as diversification within a given geographic region (Fig. 2).

Clade A (Fig. 2A; node 126) is dominated by species from the Central Hills, with the ancestor predicted to have been a Central Hills form. The lineages in this clade are confined to cool, wet habitats at higher elevations (above 800 m) and therefore are not likely to disperse among mountain ranges. However, there is evidence for one dispersal event from the Central Hills to the Rakwana Hills, and another to the Knuckles Hills, during the mid-late Miocene, resulting in the evolution of distinct species. The species that resulted from dispersal to the Rakwana and Knuckles ranges are most closely related to *P. femoralis* in the Central Hills. Of all the Central Hills taxa in Clade A, *P. femoralis* has the lowest elevational range (currently occurring down to 800 m above sea level), suggesting that the *P. femoralis* lineage may have a greater probability of dispersal to other mountain regions than lineages restricted to the cooler, higher altitudes of the Central Hills.

Clade B (Fig. 2A; node 161) shows a more complex geographic pattern. Whereas the ancestor of the clade is estimated to have been in the Central Hills, there are at least seven instances of dispersal from there to other mountain ranges, with *P. hallidayi, P. sarasinorum* and *P. cavirostris* widely distributed across both the lowlands and mountain ranges up to an elevation of 1000 m. The relationship between lineage-specific ecological characteristics and dispersal in this clade, and all others, is complex and is elaborated more fully in the Discussion below.

Clade C (Fig. 2A; node 111) is estimated to have arisen from a Central Hills ancestor, but with early dispersal to the Rakwana and Knuckles Hills and one species (*P. sordidus*) that is widespread across both lowland and mountain regions. Although the reconstructions for this clade are ambiguous, it appears that there was minor diversification within both the Knuckles and Rakwana Hills, with a complex history of migration to and from the Central Hills.

Clade D (Fig. 2A; node 44) is reconstructed as arising from a Central Hills ancestor but with migration events to the Rakwana Hills and the lowlands followed by subsequent diversifications within those ranges ([*P. decoris, P. mittermeieri, P. tanu, P. singu*] and [*P. ocularis, P. auratus*]). Additionally, a Central Hills lineage within this clade later gave rise to both a species in the Knuckles Hills (*P. hoffmanni*) as well as a lowland species (*P. hoipolloi*) – members of this clade are often found in open habitats, including anthropogenic habitats.

The common ancestor of Clade E (Fig. 2A; node 82) is estimated to have been the result of a dispersal event from the Central Hills to the Rakwana Hills. The Rakwana Hills lineage of Clade E in turn gave rise to the ancestor of two species that back-migrated to the Central Hills, and several species that now occur in the lowlands or are altitudinally widespread. Interestingly, only clade E is represented in both Sri Lanka and India. Given that it was the lowland plains of the (now submerged) Palk Isthmus that connected Sri Lanka to India during glacial sea-level lowstands, species from clade E that are now found in India most likely arose from a lowland ancestor. This suggests that a transition from cool-wet-adapted species to warm-dry-adapted ones occurred, facilitating the back migration to India during the Miocene. In fact, *P. regius,* the sister species to the Indian *Pseudophilautus*, is the only species that is

found in the dry zone of Sri Lanka. The *Ps. folicola* clade, which is the sister taxon to *P. regius* + Indian *Pseudophilautus*, is also widely distributed across the wet lowlands and all these species are small, leaf-litter dwellers.

Clade F (Fig. 2A; node 49) contains only three species, each in a different mountain range. Their MRCA is assumed, with high uncertainty, to have been a Central Hills species but all are found above 800 m in elevation on the three mountain regions, closely associated with leaf litter.

3.4. Lineage diversification in context of area and time

The gradual diversification of lineages in Sri Lanka begins during the Oligocene, following the initiation of the Tibetan-Himalayan orogenic event (ca. 30 MYA), which initiated the monsoon cycle (Licht et al., 2014; Owen and Dortch, 2014). The earliest lineages are associated with the Central Hills (Fig. 2). Early diversification of the Central Hills lineages occurs during the Mi-1 glaciation event at the Oligocene-Miocene boundary, where ice sheets formed in the Antarctic – a period conducive to spreading of cool-wet-adapted species, such as the forms that would have existed on the Central Hills. Lineages that can be attributed to Rakwana Hills arise during the early Miocene, when ephemeral Antarctic ice sheets were present (Fig. 2), which was a period of cooling that would have facilitated dispersal and diversification of lineages involving this region. Towards the mid Miocene, lowland lineages arise together with strengthening of the Asian monsoon cycles, facilitating the genesis of lowland rainforests that would have expanded the available niches for the diversifying lineages. Lineages that cross the dry lowlands and the land bridge to India originate in the late Miocene (Mi-4 glaciation event) with the strengthening of the Asian monsoons when for the first time Arctic ice sheets began to form, indicating a significant cooling event that would have facilitated the diversification by providing cool and wet niches across the warm and dry lowlands and the landbridge. Knuckles Hills lineages also arise beginning in the late Miocene (Fig. 2B). The predominant pattern of lineage diversification during the Pleistocene is across adjacent mountain ranges (i.e., Central Hills, Rakwana Hills and Knuckles Hills), with only one split within the lowland forms (Figs. 2, 3). This suggests that the increasing amplitude of climate oscillations in the late Pliocene and Pleistocene alternately allowed for migration between mountain ranges during cool, wet periods, and increased isolation among mountain ranges during warm, dry periods.

4. Discussion

4.1. Timing of diversification

Adaptive radiations often are characterized by early and rapid bursts of speciation (Schluter, 2000). We find no indication of rapid initial radiation in the Sri Lankan diversification of *Pseudophilautus*. Instead, we observe a relatively constant rate of diversification over the entire history of the clade, perhaps with a slight increase in recent diversification rate. In Sri Lanka, habitat complexity provides opportunities for ecological and allopatric speciation. These opportunities include isolated mountain ranges, a sizable tract of wet habitats, and low, dry valleys interspersing the landscape. Yet, we do not find an early-burst pattern in *Pseudophilautus*. The lack of an early-burst phase of diversification could be because climatic and habitat conditions that prevailed at the time of initial colonization (ca. 30 MYA) differed from present conditions and habitats favorable for anurans were not abundant.

The time of orogeny of the three main mountain ranges in Sri Lanka is poorly known. The few sources that refer to orogeny (Cooray, 1994; Wadia, 1945) do not provide a specific age for the Sri Lankan mountains. Therefore, it is not clear if mountainous regions were present when the common ancestor of *Pseudophilautus* colonized Sri Lanka. Interestingly, a similar diversification of *Raorchestes* inhabiting the mountainous Western Ghats of India dates to about the same time as the diversification of *Pseudophilautus* (ca. 30 MYA). The branching pattern of the *Raorchestes* phylogeny (Vijayakumar et al., 2016) also suggests a relatively constant rate of diversification and may have occurred coincident with the uplift of the Western Ghats. If the orogeny of the Western Ghats and mountains of Sri Lanka was roughly coincident, the diversification of both lineages may have tracked the creation of suitable habitats as mountains were uplifted. This scenario, while speculative, would explain the absence of an early-burst phase of diversification and is consistent with the observation of relatively slow and constant diversification rates in both groups. If the Western Ghats and Sri Lankan mountain massifs result from the same processes of orogeny, and thus time of uplift, it may explain the coincident and gradual radiations of shrub frogs in both instances. Current information is insufficient to test this hypothesis, but the coincidence in time of the two diversifications is striking and suggests that broad-scale, regional processes affected both *Raorchestes* and *Pseuophilautus* similarly.

4.2. Effects of climate on diversification

Climate certainly had a large effect on lineage geographic distribution, dispersal and the process of speciation. The Eocene-to-Oligocene transition (EOT), underscored by the Oi-1 glaciation, likely provided the opportunity for colonization of Sri Lanka. The Oi-1 glaciation was responsible for a global cooling event that resulted in species turnover in many systems (Cornette et al., 2002; Pearson et al., 2008; Liu et al., 2009; Mayhew et al., 2012; Hull, 2015). Oi-1 was a period where “full scale and permanent” glaciers arose in the Antarctic (Zachos et al., 2001). Temperatures in south Asia were mild (20–25 °C) but the climate dry, as monsoon cycles were not yet fully functional (Chatterjee et al., 2013; Hodges, 2000; Kent and Muttoni, 2008; Zhisheng et al., 2011). The Oi-1 glaciation brought about a lowstand in sea level (Kominz et al., 2008; Zachos et al., 2001) that would have facilitated migration of the most recent common ancestor of *Pseudophilautus* from mainland India to Sri Lanka over land bridges that were exposed intermittently during periods of sea-level contractions (Plyusnina et al., 2016). Since this pioneering ancestor is predicted to have been a cool-and-wet-adapted form (Fig. 2), colonization must have occurred when the environment on the land bridge and lowland areas of India and Sri Lanka were cool and wet. Indeed, many present-day species of *Raorchestes*, *Pseudophilautus* and *Mercurana* inhabit cool, wet environments (Abraham et al., 2013; Biju and Bossuyt, 2009; Biju et al., 2010) consistent with that being the ancestral state for the clade of *Pseudophilautus*.

Subsequent late Miocene (8.8 MYA) back-migration of *Pseudophilautus* from Sri Lanka to India also coincides with a “full scale and permanent” Antarctic Mi-4 glaciation during the middle to late Miocene period, which would have again exposed an intervening land bridge (Wilson et al., 2009; Zachos et al., 2001). While the regional temperature in south Asia at the time was higher than during the late Oligocene (Chatterjee et al., 2013), the Himalayan-Tibetan orogeny was well advanced, paving the way for two stable monsoon cycles annually (Chatterjee et al., 2013; Gehrels et al., 2003; Yin and Harrison, 2000). In contrast to the cool-wet-adapted ancestral *Pseudophilautus* that originally colonized Sri Lanka, the back-migrating *Pseudophilautus*, according to ancestral-state reconstructions, was a lowland form that probably was adapted to drier conditions. At present, lowland regions of India and Sri Lanka adjacent to the shallow Palk Strait that separates the two land masses are warm and dry, with scrub-forest habitats in which a single species of *Pseudophilautus* occurs on either side: *P. regius* in Sri Lanka and *P. kani* on the mainland. The ancestor of the *P. regius* + [*P. kani* + *P. amboli* + *P. wynaadensis*] clade was likely a similarly dry lowland-adapted species.

The period between the initial migration (Oi-1 glaciation, 31 MYA) and subsequent back-migration (Mi-4 glaciation; 8.8 MYA) coincided

with intensification of the monsoons (Briggs, 2003; Voris, 2000; Wilson et al., 2009) and seems to have been a period favorable for diversification of *Pseudophilautus* on Sri Lanka. Most extant species (ca. 45) of the *Pseudophilautus* radiation are of late Miocene origin. Similar climatic conditions may have triggered the diversification of Indian *Raorchestes,* of which the majority of species similarly originated during the Miocene (Vijayakumar et al. 2016).

The diversification rate of *Pseudophilautus* in Sri Lanka shows a slight increase in the Pleistocene, with about ten recognized species originating during that period. The Pleistocene, with repeated cycles of expanding and contracting forests, may have facilitated rapid speciation in both Sri Lankan *Pseudophilautus* and Indian *Raorchestes* (Vijayakumar et al., 2016) through population expansion and dispersal during periods of favorable climate and population isolation and divergence during unfavorable periods. These same influences would have shifted the ranges of some Sri Lankan *Pseudophilautus* along altitudinal gradients while isolating others in lowland forest fragments (Deraniyagala, 1989, 1997, 2007).

4.3. The geography of diversification

The ancestor of *Pseudophilautus* and subsequent ancestral forms as recently as the early Miocene (ca. 20 MYA) reconstruct as Central Hills forms. Other geographic areas (Lowlands, Knuckles Hills and Rakwana Hills) were colonized from the Central Hills, indicating that the Central Hills is an important center of persistence and source of migrants. The Central Hills is not a climatically and topographically uniform area. Due to its size (the land area above 700 m in the Central Hills is 3498 km^2, which is substantially larger than both the Rakwana Hills, 182 km^2, and Knuckles Hills, 565 km^2) and its location at the center of the island, the Central Hills mediates rainfall over the island during the two major monsoons. The stronger monsoon sweeps in from the southwest, peaking in June and July; the weaker approaches from the northeast, peaking in October and November. This pattern results in a much drier northern and eastern part of the island in comparison to southern and western regions (Domroes et al., 1998). Furthermore, the valleys and peaks of the Central Hills, many of them arranged in a centrally placed, anchor-like formation, form a topographically complex area, providing opportunities for allopatric divergence within the massif.

If the orogeny in Sri Lanka did not precede the diversification event, a scenario in which Central Hills forms first and Knuckles and Rakwana Hills form later can explain the existing pattern of diversification. Another, less plausible scenario is that Central, Rakwana and Knuckles Hills were once part of a single mountain range but subsequently became isolated from one another by erosion of the Kalu and Mahaweli valleys. This scenario, however, requires that early lineages of *Pseudophilautus* go extinct on the Rakwana and Knuckles Hills, and it does not explain why the oldest lineages in the diversification are in the Central Hills.

The geographic distribution of sister lineages in the Sri Lankan radiation does not show diversification clustered primarily within geographic areas or among areas. Instead, cladogenic events within and between areas occur at about equal frequencies across the tree. Excluding the nodes for which geographic state was ambiguously reconstructed, there are 22 instances in which sister lineages arising from a node are in different areas and 26 in which sister lineages are in the same area. This suggests that speciation occurred both within and between areas and, importantly, that there were periods when climatic conditions allowed for migration between what are now climatically and geographically isolated mountain ranges. Species ranges likely expanded during favorable climatic periods and contracted during unfavorable periods, resulting in repeated instances of dispersal between mountain ranges followed by isolation. Thus, dynamic changes in species distribution and dispersal, correlated with climate fluctuations, likely were the main drivers of current species distribution and diversity.

4.4. Patterns of historical dispersal are correlated with species natural history

Species assemblages in most regions are composites of species drawn from diverse clades in the phylogeny, and there is an interesting, but perhaps unsurprising, correlation among life history, current distribution and the likelihood of historical dispersal among mountain ranges. Overall, lineages that have lower minimum elevational ranges are more likely to be sources of dispersal. The lower minimum elevational range is often associated with apparent changes in natural history, such as adaptation to live among streambed and streamside boulders, or exploitation of moist microhabitats in open areas.

Species in Clade A (Fig. 2, node 126) display little dispersal among mountain ranges. These species are restricted to higher elevation forests, with only one group (the “femoralis-mooreorum” group; Fig. 2, node 120) having dispersed among mountain ranges. Species in Clade A lay clutches on leaves (as opposed to soil nesting in all other clades), and the clutches have a long pre-hatching period (37–49 days in *P. femoralis*; Bahir et al. 2005). This derived reproductive mode within *Pseudophilautus* may restrict members of this clade to perenially cool, wet habitats at higher elevations, thus making it less likely for lineages to disperse among mountain ranges.

In contrast to Clade A, many sister species in Clade B (Fig. 2, node 161) are distributed across adjacent mountain ranges. Several dispersal events must be inferred to explain their distribution. This pattern suggests that species in Clade B have greater dispersal ability and/or less restrictive habitat requirements than species in Clade A. Interestingly, some species in Clade B are found in boulder habitats in proximity to streams and streambeds (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura and Manamendra-Arachchi, 2005; Fig. 2, node 152 and *P. hallidayi*). Deep crevices among large boulders provide stable cool and wet microclimates that would facilitate population persistence in otherwise dry habitat. Boulders, streambeds and gallery forests may have provided important corridors for dispersal among mountain ranges and into the wet lowlands for this group.

Within Clade C (Fig. 2, node 111), there are three subclades that display different ecologies and likelihood of dispersal between mountain ranges. *Pseudophilautus microtympanum* and *P. steineri* are range-restricted, high-altitude grassland and forest inhabitants (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura and Manamendra-Arachchi, 2005). Their dispersal between mountain ranges could only occur when cool, wet habitats dominated the lowlands. In contrast, species in the *sordidus* group are widespread and contain species capable of living at lower elevations in streamside boulders and vegetation (Manamendra-Arachchi and Pethiyagoda, 2005). These species are likely to have a greater capacity to disperse along streams and gallery forests. Species in the “silus” group (*P. silus*, *P. cf. silus* and *P. fulvus*; Fig. 2, node 93) are found at low-to-mid elevations in closed canopy forests as well as cardamom plantations and other human modified, closed habitats (Manamendra-Arachchi and Pethiyagoda, 2005). Dispersal for this group would be facilitated by slightly wetter conditions than are present currently, and dispersal could occur along gallery forests and streambeds. Finally, *P. reticulatus* is found in the canopy of tall, mature, lowland forests. While it is restricted to the wet southwest of Sri Lanka (Manamendra-Arachchi and Pethiyagoda, 2005) and its sister species, *P. papilosus,* is restricted to the Rakwana Hills (Manamendra-Arachchi and Pethiyagoda, 2005), together they are distributed along an altitudinal gradient from the Rakwana Hills to lowland rainforests.

Species in Clade D (Fig. 2, node 44) can be considered to comprise three subclades that are ecologically distinct and possess different dispersal abilities. The “hoipolloi-asankai” group (*P. asankai, P. pleurotaenia, P. hoffmanni* and *P. hoipolloi*; Fig. 2, node 41) have a wide distribution and are associated with open habitats such as forest gaps, plantations and home gardens (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura and Manamendra-Arachchi,

2005). They are represented on all three hill ranges and extend across the lowland wet zone. Since affiliates of this clade have not dispersed into the lowland dry zone, they are not well adapted to warm, dry conditions and may be exploiting cool, wet microhabitats within open habitats. The subclade containing *P. ocularis* (narrowly distributed above 1000 m in the Rakwana Hills) and *P. auratus* (found from 500 to 1200 m; Fig. 2, node 34) constitutes species that live under forest cover. Hence, compared to the "hoipolloi-asankai" group (Fig. 2, node 41), this group shows lower vagility. The "decoris-tanu" group (*P. tanu, P. singu, P. mittermeieri* and *P. decoris*; Fig. 2, node 31) likewise is found only in closed forest and has a limited distribution across the Rakwana Hills (*P. decoris*) and predominantly the lowlands (the other three species). The "hoipolloi-asankai" group within Clade D appears to have evolved the ability to exploit cool, wet habitats within a warm, dry matrix, and therefore its species have greater dispersal ability.

Members of Clade E (Fig. 2, node 82) are predominantly lowland inhabitants showing the greatest dispersal abilities and apparent adaptation to warm and dry conditions. This includes the "regius-kani" group (*P. regius, P. amboli, P. wynaadensis* and *P. kani*; Fig. 2, node 63) containing warm-dry-adapted forms, which have facilitated even the back-migration to India across the warmer and drier lowlands. The ability of these species to utilize wet microhabitats, marshes, paddy fields and riverine corridors (Manamendra-Arachchi and Pethiyagoda, 2005; Meegaskumbura et al., 2007, 2009; Meegaskumbura and Manamendra-Arachchi, 2005) would have facilitated their dispersal.

Species in Clade F (Fig. 2, node 49) are characterized by small body sizes and are restricted to elevations above 800 m. They are characterized by small distributions and show limited dispersal capabilities. These are forest cover and leaf litter-dependent forms (Manamendra-Arachchi and Pethiyagoda, 2005), which, coupled with elevational constraints, seem to have low dispersal abilities.

4.5. The role of terrestrial direct development in the diversification

Diversification of *Pseudophilautus* into a wide range of terrestrial habitats likely was facilitated by their derived reproductive mode of terrestrial direct development, which evolved at least 25 million years ago (Fig. 2). Within Rhacophoridae, direct development has evolved 2 or 3 times (this study supports 3 times), and in each instance the corresponding direct-developing clade is substantially more diverse than its indirect-developing (gel-nesting) sister clade (Meegaskumbura et al., 2015). A vast majority of species-rich, direct-developing anuran diversifications occur in perennially wet habitats, and this is true for *Pseudophilautus* as well. Only one species (*P. regius*) is found in the drier lowlands, and even there it is associated with pockets of wet habitat.

The clade of Indian *Pseudophilautus* that is nested within the Sri Lankan diversification shares most features of the island group, including direct development. Yet, it is species poor in comparison to many other clades that diversified extensively since the late Miocene. The impressive taxonomic diversity (at both species and supra-specific levels) and diversity of habitats occupied by anurans of the Western Ghats (Abraham et al., 2013; Biju et al., 2008, 2009, 2011, 2013, 2014; Biju and Bossuyt, 2009; Bocxlaer et al., 2010) suggest that ecological opportunity for *Pseudophilautus* back-migrating to India may have been limited.

In contrast, the initial, earlier migration to Sri Lanka may have provided abundant ecological opportunity to those early colonizers in the form of habitat unexploited by other anurans. Yet, and surprisingly, we do not find an early burst of diversification. Rather we find a stately pace of diversification whose environmental or internal drivers remain obscure. The current exceptional diversity of *Pseudophilautus* within Sri Lanka certainly owes much to the long period of time available for diversification (31 MYA), abundant ecological opportunity (complex habitat and fewer competing lineages when compared to the mainland) and an associated key innovation (terrestrial direct development).

5. Conclusions

The remarkably extensive diversification of *Pseudophilautus* in Sri Lanka shows several key features. It is an ancient diversification that began at the end of the Oligocene, probably facilitated by climatic events at the Oligocene-Miocene transition. Contrary to other island radiations, this diversification lacks an early-burst phase, suggesting perhaps that suitable habitats became available gradually over time rather than being present and available at the time of colonization. The species that are adapted to lower minimum elevational ranges (i.e., warmer and drier conditions) are more likely to be sources of dispersal among mountain ranges when compared to the elevationally restricted species. During climatically favorable periods, the valleys between mountain ranges were crossed by species giving rise to montane communities made up of members derived from disparate clades. A lineage nested within the Sri Lankan diversification back-migrated to India during the late Miocene, but this clade is depauperate, possibly due to the prior occupation of mainland niches by other anuran lineages, including direct-developing *Raorchestes*. Terrestrial direct development may constitute a key innovation that facilitated diversification by allowing species to breed away from water sources and thus to exploit a wide array of terrestrial habitats. The lowland lineages in Sri Lanka are derived from montane lineages and thus the mountains of Sri Lanka acted as both species pumps and refuges throughout the 31 million years of evolution. Together, these features highlight the importance of montane regions in generating and sustaining the remarkable diversity of Sri Lankan shrub frogs through time.

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Appendix A. Supplementary material

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